Postexposure Prophylaxis against Experimental Inhalation Anthrax

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Inhalation anthrax is a rare disease that is almost invariably fatal. This study determined whether a prolonged course of postexposure antibiotics with or without vaccination would protect monkeys exposed to a lethal aerosol dose of *Bacillus anthracis* when the antibiotic was discontinued. Beginning 1 day after exposure, groups of 10 animals were given penicillin, ciprofloxacin, doxycycline plus vaccination, vaccination alone, or saline. Antibiotics were administered for 30 days and then discontinued. Vaccine was given on days 1 and 15. Two animals died of causes other than anthrax and were not included in the statistical analysis. Nine of 10 controls and 8 of 10 animals given only vaccine died. Each antibiotic regimen completely protected animals while on therapy and provided significant long-term protection upon discontinuance of the drug (penicillin, 7of 10 survived, P<.02; ciprofloxacin, 8 of 9 survived, P<.0002). Protection against rechallenge was provided by combining postexposure antibiotic treatment with vaccination.

Anthrax is a zoonotic infection caused by *Bacillus anthracis*. Humans become infected by contact with infected animals or contaminated animal products. Anthrax begins by introduction of the spore through skin, producing cutaneous anthrax; the gastrointestinal tract, causing gastrointestinal anthrax; or the respiratory tract, causing inhalation or mediastinal anthrax. Inhalation anthrax is extremely rare, with ~30 cases reported in this century, most often associated with industrial exposure to spores [1]. The disease has been almost uniformly fatal because of the difficulty in establishing the diagnosis and the rapid progression of the disease. Previous experimental studies demonstrated that treatment with penicillin for 5 to 10 days, beginning 1 day after aerosol exposure of monkeys, was protective during drug therapy, but animals died when the antibiotic was discontinued [2]. Long-term protection was afforded only by combining penicillin therapy with postexposure vaccination. Recent events in the Gulf War heightened the awareness of the possibility that anthrax could be used as a biological weapon. For this reason, we determined whether a more prolonged course of antibiotic therapy alone or with vaccination, begun after an aerosol exposure, could protect animals from inhalation anthrax.

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The experiments were carried out under the guidance of the Veterinary Medicine Division in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication 86-23. 1985. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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Materials and Methods

Bacterial strain preparation, animals, and aerosol exposure. *B. anthracis* Vollum 1B spores were prepared as previously described, except for the omission of centrifugation through Renografin gradients [3]. Spores were diluted in sterile water, heated at 60°C for 45 min. and then dept on ice. Rhesus monkeys *(Macaca mulatta)* of both sexes (5.8-13.0 kg) anesthetized with tiletamine/zolasepam (3 mg/kg; A.G. Robins, Richmond, VA) were exposed in a head-only chamber to an aerosol generated with a Collision nebulizer. The mass median diameter of the particles generated was 1.2 µm as determined by cascade impacter and particle sizer (model 3310; TSI, St. Paul). The concentration of spores in the aerosol was determined during each exposure using an all-glass impinger. The minute respiratory volume was measured on each animal immediately before exposure. In the first challenge experiment, animals wee exposed to an inhaled dose of $4.0 \pm 1.6 \times 10^5$ spores (mean ± SD), corresponding to ~ 8 LD_{50} [2] (unpublished data). Survivors from the first experiment were rechallenged with an inhaled dose of $2.6 \pm 1.4 \times 10^6$ spores (50 LD_{50}).

Experimental groups. Animals wee randomly distributed by sex and weight into six groups of 10 animals each. (1) Controls were given saline intramuscularly (im) every 12 h, beginning 1 day after exposure. (2) Procaine penicillin G was given im at a dose of 180,000 units (0.6 mL) every 12 h, beginning 1 day after exposure and continuing for 30 days: (3) ciprofloxacin, 125 mg (in 5 mL H_20): (4) doxycycline. 30 mg (6mL);(5)doxycycline, 30 mg (6 mL). Plus vaccination (0.5 mL of human anthrax vaccine [Michigan Department of Public Health, lot FAV001] on days 1 and 15 after aerosol exposure).

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(6) The vaccination group received 0.5 mL of the human anthrax vaccine on day 1 and, if still alive, on day 15 after aerosol exposure and water by orgiastic tube every 12 h, beginning 1 day after exposure and continuing for 30 days.

Animals given ciprofloxacin, doxycycline, or vaccination were anesthetized with tiletamine/zolazepam (3 mg/kg) so medication could be given by orogastic tube. After 30 days of treatment, antibiotics were discontinued. Survivors were rechallenged 130\1-142 days after the initial exposure together with 5 new saline control monkeys.

Clinical, microbiologic and pathologic studies. Daily blood cultures were obtained from the saline controls and the group given only vaccine until death or for 14 days. In the antibiotic-treated groups, blood was cultured every other day until 80% of the controls died, then twice weekly until day 30, then every other day until ~day 60, and then once a week until rechallenge. Blood from animals not given antibiotics was collected in an Isolator 1.5 (Du Pont, Wilmington, DE) and cultured in 10-fold dilutions in triplicate on trypticase soy agar. Blood from antibiotic-treated animals collected in an Isolator 1.5 was cultured undiluted and at a 1:100 dilution on a trypticase soy agar. In addition, 1 mL was cultured in a BACTEC Peds Plus bottle (Becton Dickinson, Towson, MD). Blood obtained before and at various times after challenge was analyzed for IgG antibodies to the anthrax protective antigen by ELISA [4].

Moribund animals were killed by deep anesthesia (tiletamine/zolazepam. 6 mg/kg) and exsanguination. All animals were autopsied. A diagnosis of anthrax was confirmed in all animals by isolating *B. anthracis* from the blood. In some cases, organs were cultured quantitatively. In all deaths in which antemortem blood cultures were negative, cultures were done of blood, spleen, lung, liver, intrathoracic lymph nodes, and brain.

Antibiotic sensitivity testing and serum levels. MICs of the *B. anthracis* Vollum 1B strain were determined in Mueller-Hinton broth dilutions using an inoculum of 2.5-3.0 X 10^5/mL in tubes and in a microtiter format. The MIC was 0.08 µg/mL for penicillin, 0.08 µg/mL ciprofloxacin, and 0.02 µg/mL for doxycycline. The MBC was 0.32 µg/mL for penicillin and 0.08 µg/mL for ciprofloxacin. Peak serum levels were determined for 1 h (ciprofloxacin) or 2 h (penicillin and doxycycline) after a dose. Trough levels were measured 12 h after a dose for all drugs.

Procaine penicillin G, penicillin G potassium used as a reference standard, and doxycycline monohydrate suspension were purchased from Pfizer (New York). Powdered doxycycline hyclate for a reference standard was a gift of Pfizer (Groton, CT). Ciprofloxacin tablets and powdered ciprofloxacin for a reference standard were gifts of Miles Pharmaceuticals (West Haven, CT).

Statistical analysis. The significance of the difference in survival between the experimental and control groups was determined using Fisher's exact test, two-tailed.

Results

Description of disease in controls. Nine of the 10 control animals exposed to an inhaled dose of 8 LD_{50} died 3-8 days after challenge (mean ± SE1.1). The animals were ill for 1 to 4 days before death, demonstrating decreased spontaneous activity, weakness, and anorexia. One animal had a single seizure on the day of death and was found on autopsy to have meningitis. Respiratory distress was observed in only 1 animal. Bacteremia at levels of 10-10^5 cfu/mL was present for a mean of 1.8 ± 0.9 days before death. Terminal bacteremias in 8 of the 9 animals that died varied 10^4 to 10^9 cfu/mL. The 1 animal that survived never had a positive blood culture.

Antibiotic serum levels. The mean peak and trough serum levels for each of the antibiotics did not vary significantly when measured on days 5, 9, 20, and 30. The peak levels were at least 10 ties the MIC for all antibiotics, and the trough levels varied from 1 times the IC for ciprofloxacin to 1-10 times for penicillin and doxycycline [5].

Effect of postexposure treatment on survival. Survival of the various treatment groups is shown in table 1 and figure 1. Eight of 10 animals treated with vaccination alone died. The time to death and clinical autopsy findings did not differ from untreated controls. One of the two vaccinated animals that survived had persistently negative blood cultures. The other had positive blood cultures on days 5, 11, and 12 at low levels of 10-20 cfu/mL with negative blood cultures thereafter.

We observed significant protection against death in each of the antibiotic-treated groups. All animals in the penicillin group survived the 30 days of treatment, during which their blood cultures were negative. Three of 10 animals died of anthrax on days 9, 12, and 20 after penicillin was stopped.

One animal given ciprofloxacin died 5 days after exposure from an aspiration pneumonia 24 h after the inadvertent introduction of drug into the trachea. All antemortem blood cultures were negative for anthrax as were postmortem cultures of blood, lung, liver, spleen, and brain.

Table I. Survival after postexposure treatment of inhalation anthrax.

Treatment	Anthrax deaths	*P* vs. control
Control untreated	9/10	
Vaccine alone	8/10	>.1
Penicillin	3/10	< .02
Ciprofloxacin	1/9[=]	< .002
Doxycycline	1/10	< .002
Doxycycline + vaccine	0/9[†]	< .0002

[=] One animal died 5 days after exposure from aspiration pneumonia, had no evidence of anthrax at autopsy, and was excluded from analysis.

† One animal died 6 days after discontinuing doxycycline with no evidence of anthrax on autopsy. Cause of death remains unknown; the animal was excluded from statistical analysis.

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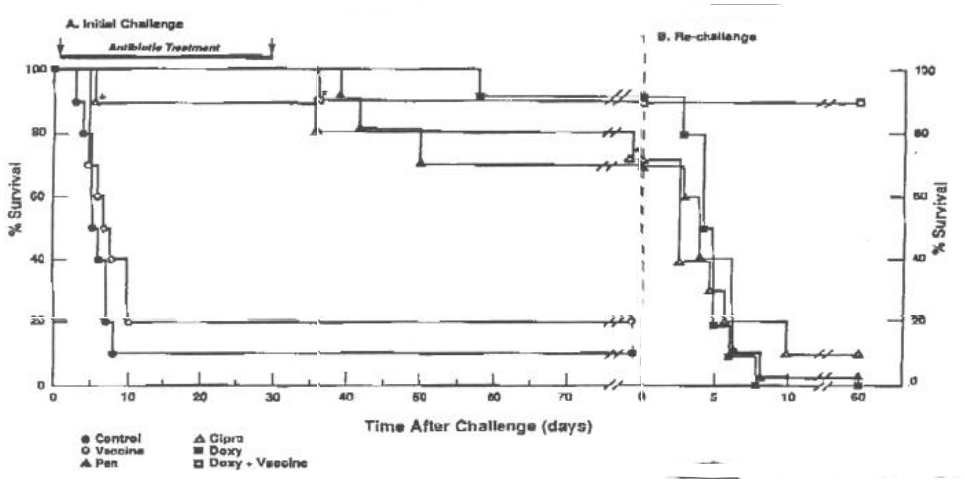

Figure 1. Effect of postexposure antibiotic treatment antibiotic treatment and vaccination on survival for inhalation anthraxand subsequent rechallenge. **A.** Groups of 10 animals were exposed to aerosol of anthrax spores on day 0 and were untreated (control), given vaccine only on days 1 and 15, or treated with penicillin (pen), ciprofloxacin (cipro), doxycycline (doxy), or doxy plus vaccination. Antibiotics were given from days 1 to 30. **B.** Survivors wee rechallenged by aerosol on days 131-142 (day 0, B). percentage survival is plotted against day after initial challenge (A) or rechallenge (B). *, 3 animals that died of causes other than anthrax.

Pathologic analysis showed an aspiration pneumonia with no evidence of anthrax in any organ. This animal was Eliminated from analysis, as no assessment of the effectiveness of antibiotic treatment on long-term survival was possible. All other ciprofoxacin-treated animals survived the 30 days of treatment with negative blood cultures. One animal died of anthrax 6 days after antibiotic was stopped. Another animal in the ciprofloxacin group died 73 days after antibiotic was discontinued. Autopsy revealed no evidence of anthrax either by culture or histologically. This animal died of urethral obstruction due to rubbery plugs (concentrations) in the proximal urethra and bladder and was considered to be a survivor of the anthrax challenge.

In the group treated with doxcycline alone, all animals survived during therapy and had negative blood cultures. One animal died of anthrax 28 days after treatment was stopped.

None of the animals treated with doxycycline plus postexposure vaccination died of anthrax. One animal in this group died 6 days after discontinuance of doxycycline but had no evidence of anthrax on autopsy by either culture or histologically.. Mild myocardial degeneration was observed but the exact cause of death could not be determined. The effect of treatment on long-term survival from anthrax could not be evaluated and this animal was eliminated from statistical analysis.

Animals that survived the aerosol challenge were examined for evidence of an immune response 131-142 days after exposure by measuring antibody to the protective antigen component of anthrax toxin. No surviving animals given vaccine in addition to doxycycline all developed a fourfold or greater rise in antibody. The two surviving animals given vaccine alone also developed an antibody response.

Resistance of surviving treated animals to rechallenge. Significant protection against rechallenge of the survivors occurred in the group vaccinated and treated with doxycycline, with 9 of 9 animals surviving (figure 1). These animals remain free of disease 1 year after rechallenge. No significant protection was afforded by antibiotic treatment alone (penicillin, 0/7 survived; ciprofloxacin, 1/7 survived; doxycycline, 0/9 survived).

Discussion

The clinical and pathologic findings we observed after aerosol exposure to anthrax spores are consistent with those previously reported in nonhuman primate studies [6, 7]. The observations are strikingly similar to those reported in cases of human inhalational anthrax [1, 8[, confirming the relevance of this experimental model [2, 6, 7]. The most striking pathologic findings were intrathoracic hemorrhagic lymphadenopathy, mediastinitis, and meningitis.

One control animal survived the initial aerosol challenge and appears not to have become infected, as blood cultures were negative and in immune response never developed.

There were three deaths that were not due to anthrax. One ciprofloxacin-treated animal died of an aspiration pneumonia, 1 animal in the doxycycline plus vaccine group died 5 days after discontinuing the antibiotic and the cause of death could not be established, and 1 animal in the ciprofloxacin group died 73 days after antibiotic was discontinued because of urethral obstruction due to rubbery concretions. The relationship of the latter finding to he crystalluria induced by ciprofloxacin in nonhuman primates [9] is unclear., as the ciprofloxacin had been stopped for >2 months.

Critical to the rational treatment of inhalation anthrax is an understanding of the initial pathogenesis of the disease. Studies by Ross [10] suggest that inhaled spores are phagocytosed by alveolar macrophages and transported to the regional lymph nodes where they germinate to vegetative bacilli. However,

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some of the inhaled spores do not germinate and remain dormant within the lung for extended periods. Henderson et al.[2] demonstrated that 2 days after inhalation of spores by monkeys, 15%-20% of the initially retained dose of spores was still present in the lung. The significance of this for therapy of anthrax was initially appreciated by Barnes [11], who stated in reference to penicillin that spores may persist in the tissues and germinate after the level in blood falls. The findings by Henderson et al. [2] that animals treated for 5 or 10 days with penicillin died when the antibiotic was discontinued are consistent with this concept.

Our experiments clearly demonstrate that more prolonged antibiotic treatment for 30 days results in statistically significant long-term survival after discontinuance of treatment. Seven of 10 penicillin-, 8 of 9 ciprofloxacin-, and 9 of 10 doxycycline-treated animals survived. This result supports the hypothesis that treatment with antibiotics alone will be successful if the treatment continues until the level of retained persistent spores falls to less than the infectious dose. However, the five anthrax deaths in the antibiotic-treated animals, particularly the animal that died 58 days after exposure, directly support the concept that spores persist for prolonged periods in the host. The present data, taken together with the previous report of treatment failure with a short course of antibiotics [2], suggest that an even more prolonged course of antibiotics might have prevented all deaths from anthrax.

The results also showed that complete, long-term survival, after discontinuance of antibiotics, occurred when postexposure antibiotic treatment was combined with vaccination, confirming previous reports [2, 12]. Survival rate in these animals did not differ statistically from that of animals treated with antibiotics alone.

No animals treated with penicillin, ciprofloxacin, or doxycycline alone developed evidence of an immune response to anthrax. This suggests that antibiotic treatment, begun early after exposure, prevented the infection from fully developing. The only animals that seroconverted after the aerosol challenge were those that had been vaccinated. A serologic response is observed in humans who recover from established clinical anthrax after treatment [4, 13] and in monkeys after vaccination [14] (unpublished data).

Development of an immune response was found to predict resistance to rechallenge. The only animals resistant to a second aerosol challenge were those that had been vaccinated and had seroconverted (figure 1). Animals protected against the initial infection by antibiotic treatment did not develop an effective immune response and were susceptible to reinfection. This agrees with a previous report where animals treated after exposure with antibiotics and hyperimmunization with five doses of vaccine and that survived were protected upon rechallenge [12]. The protection afforded by vaccination before exposure is to be expected, on the basis of prior reports [14, 15] and our experiments (unpublished data).

Thus, these results suggest that therapy for an unimmunized person exposed to an aerosol of anthrax spores should consist of long-term suppressive antibiotics. Vaccination may provide an additional degree of protection against relapse after antibiotic treatment and would protect against a subsequent exposure.

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